Spurious Underestimation of Results for Digoxin Radioimmunoassay with a Commercial Kit

Lynn R. Witherspoon, Stanton E. Shuler, and Meredith M. Garcia

Estimates of digoxin in serum with a commercial radioimmunoassay kit (Schwarz/Mann) occasionally result in more counts in the patient's "bound" fraction than in the "free" fraction for the zero standard. This phenomenon has been investigated in 26 serum samples obtained from patients who were ingesting known amounts of digoxin. The problem appears to relate to environmental differences in at least the zero standard and affects results when serum digoxin concentrations are less than 0.8 μg/liter. Apparently dose estimates are more accurate with other methods or by calculating results after omitting the zero standard.

Radioimmunoassay of digoxin was described in 1969 by Smith et al. (1), utilizing antisera directed against a human serum albumin/digoxin hapten and tritiated digoxin. "Bound" and "free" fractions were separated with charcoal. These antisera appeared to be directed largely against determinates in the C-D rings of the digoxin molecule (2). Substitution of aromatic hydrocarbons for the glycoside at the 3-O position in the steroid nucleus permits the introduction of 125I. Use of such a substituted molecule as the labeled ligand minimally affects antiserum binding, improves assay sensitivity, and avoids the problems of liquid scintillation counting (3). In a commercial assay kit (Schwarz/Mann) an iodinated 3-O succinyl tyrosine digoxin analog is used for the labeled ligand.

Since it was first reported in 1972 by Davidson (4) we also have occasionally observed patients' samples in which binding of labeled ligand by antisera exceeded that of the zero standard by the Schwarz/Mann assay. In addition, acceptable analytical recovery of added digoxin from these samples could not be demonstrated. Apparently there were some patients receiving digoxin whose concentration of it in serum could not be measured with this assay. Furthermore, it raised the question of significant underestimation of concentrations in the serum of patients receiving therapeutic amounts of digoxin.

We have examined this phenomenon in some patients who were originally identified when estimation of digoxin concentration was attempted by the Schwarz/Mann assay and the counts in the "bound" fraction exceeded those in the zero standard ($B/B_0 > 100\%$).
Methods

We studied 26 serum samples from 24 such patients. Thirty-nine serum samples from patients receiving no digoxin were also studied.

Assays

Digoxin radioimmunoassay "kits" were obtained from Schwarz/Mann, Orangeburg, N.Y. 10962; Abbott Laboratories, North Chicago, Ill. 60064; and Corning Medical Diagnostics, Medfield, Mass. 02052. The Schwarz/Mann assay utilizes an iodinated digoxin succinyl tyrosine analog as the labeled ligand, provides the standards in human serum, and separates the "bound" and "free" fractions by charcoal. The Abbott assay utilizes an iodinated digoxin tyramine analog, provides the standards in human serum, and separates the "bound" and "free" fractions with polyethylene glycol 8-Anilino-1-naphthalene sulfonic acid is included to inhibit protein binding of endogenous digoxin. The Corning assay utilizes an iodinated digoxin tyrosine analog as the labeled ligand, and provides the standards in pooled plasma. The antiserum is covalently bound to glass beads, the "bound" fraction separating on this solid phase. We followed the assay protocols as described in the manufacturers' literature. Standards were diluted with the zero standard to obtain 0.2–0.25 μg/liter.

Patients

The medical history was obtained, including digoxin dosage schedule, underlying medical disorders (in particular renal status), and all medications received. Serum total protein and albumin concentrations were measured. Serum samples were collected approximately 24 h after the last oral dose of digoxin, except for six patients who received their last digoxin dose more than 24 h earlier. All patients were receiving digoxin in various dosages on a regular basis.

Studies

Standardization. All three commercial standards were estimated in all three assays. In addition, a digoxin (Lanoxin; Burroughs Wellcome and Co., Research Triangle Park, N.C. 27709) standard was prepared in human serum and estimated in all three assays.

Parallelism. Three serum samples containing a high concentration of digoxin were diluted serially with both Schwarz/Mann zero standard and with digoxin-free sera. These serial dilutions were assayed for apparent digoxin concentration by using the Schwarz/Mann assay.

Estimation of unknown digoxin concentration in patient serum. The apparent digoxin concentrations were estimated, for the 26 serum samples obtained from the 24 patients receiving digoxin, in all three assays by counting the "bound" fraction.

Nonspecific binding. Nonspecific binding of the 26 serum samples obtained from patients receiving digoxin was estimated by the Schwarz/Mann method by adding labeled ligand but no antiserum to patients' samples, incubating, separating "bound" from "free" fractions, and counting the "bound" fraction. This also served to exclude gross misclassification of "free" as "bound" in the patient samples.

Analytical recoveries. Lanoxin was added to 10 of the 25 samples obtained from patients receiving digoxin to yield additions of 1.0 and 2.5 μg of digoxin per liter. Lanoxin volumes were 4 and 10 ml/ml of serum, respectively. Recovery was estimated for these samples in all three assays.

\[1\] A table containing this information may be obtained from the authors.

Fig. 1. Standard displacement curves for three commercial standards and Lanoxin in the three assays evaluated.

Estimation of apparent digoxin concentration in digoxin-free sera. Apparent digoxin concentrations by all three assays were estimated for 39 serum samples obtained from patients not receiving digoxin and compared with the binding observed for the zero standard provided by the manufacturer.

Controls. Estimates were obtained by all three assays for two commercial (Ortho Diagnostics, Inc., Raritan, N.J. 08869) and two in-house pools of serum, the digoxin concentrations being approximately 0.8, 0.8, 4.0, and 4.5 μg/liter.

Calculations

Initial calculations were based on the standard dose/response curve expressed as logit B/B₀ vs. log dose in micrograms per liter (5). This data transformation was demonstrated to fit the raw data over the range of 0.25–5.0 μg/liter for all three assays. The Schwarz/Mann data were recalculated as logit "bound"/"total" (B/T) vs. log dose, excluding the zero standard. Unknown dose estimates were provided by computer calculation, with use of a weighted least-squares linear regression (5).

Recoveries were calculated as the amount of digoxin measured divided by the amount of digoxin added plus the amount of digoxin assayed in the sample before the addition.
Table 1. Results of Digoxin Assay with Three Kits

<table>
<thead>
<tr>
<th>Pt #</th>
<th>Digoxin (µg/liter) (B/B₀)</th>
<th>Recalculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Schwarz/Mann</td>
<td>Abbott</td>
</tr>
<tr>
<td>1A</td>
<td>&lt;0.25/97</td>
<td>0.61/85</td>
</tr>
<tr>
<td>1B</td>
<td>&lt;0.25/94</td>
<td>0.64/84</td>
</tr>
<tr>
<td>2</td>
<td>0.42/86</td>
<td>0.90/78</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.25/93</td>
<td>0.61/85</td>
</tr>
<tr>
<td>4</td>
<td>0.37/88</td>
<td>0.61/85</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.25/107</td>
<td>&lt;0.25/96</td>
</tr>
<tr>
<td>6</td>
<td>0.36/88</td>
<td>0.77/81</td>
</tr>
<tr>
<td>7</td>
<td>&lt;0.25/98</td>
<td>0.47/89</td>
</tr>
<tr>
<td>8</td>
<td>0.25/100</td>
<td>0.50/88</td>
</tr>
<tr>
<td>9</td>
<td>&lt;0.25/95</td>
<td>0.39/90</td>
</tr>
<tr>
<td>10</td>
<td>&lt;0.25/107</td>
<td>&lt;0.25/104</td>
</tr>
<tr>
<td>11</td>
<td>&lt;0.25/97</td>
<td>0.25/96</td>
</tr>
<tr>
<td>12</td>
<td>&lt;0.25/212</td>
<td>0.28/98</td>
</tr>
<tr>
<td>13</td>
<td>&lt;0.25/103</td>
<td>0.25/97</td>
</tr>
<tr>
<td>14</td>
<td>0.25/91</td>
<td>0.39/90</td>
</tr>
<tr>
<td>15</td>
<td>&lt;0.25/108</td>
<td>&lt;0.25/96</td>
</tr>
<tr>
<td>16</td>
<td>0.31/90</td>
<td>0.64/86</td>
</tr>
<tr>
<td>17</td>
<td>&lt;0.25/99</td>
<td>0.25/95</td>
</tr>
<tr>
<td>18</td>
<td>&lt;0.25/98</td>
<td>0.56/86</td>
</tr>
<tr>
<td>19</td>
<td>&lt;0.25/99</td>
<td>0.37/91</td>
</tr>
<tr>
<td>20</td>
<td>0.36/88</td>
<td>0.69/83</td>
</tr>
<tr>
<td>21 A</td>
<td>&lt;0.25/112</td>
<td>&lt;0.25/101</td>
</tr>
<tr>
<td>21 B</td>
<td>0.27/91</td>
<td>0.75/82</td>
</tr>
<tr>
<td>22</td>
<td>&lt;0.25/107</td>
<td>&lt;0.25/100</td>
</tr>
<tr>
<td>23</td>
<td>0.25/108</td>
<td>0.35/92</td>
</tr>
<tr>
<td>24</td>
<td>&lt;0.25/104</td>
<td>&lt;0.25/96</td>
</tr>
</tbody>
</table>

Table 2. Analytical Recovery of Added Digoxin (B/B₀ Calculation) for Three Kits

<table>
<thead>
<tr>
<th>Digoxin added, µg/l</th>
<th>Per cent recovery (±1 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Schwarz/Mann</td>
</tr>
<tr>
<td>1</td>
<td>109 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>119 ± 7</td>
</tr>
</tbody>
</table>

Table 3. Binding in Sera from Patients Not Taking Digoxin (n = 39), as Measured with Three Kits

<table>
<thead>
<tr>
<th>Calculation B/B₀ (±1 SD)</th>
<th>Schwarz/Mann</th>
<th>Abbott</th>
<th>Corning</th>
</tr>
</thead>
<tbody>
<tr>
<td>105 ± 2%</td>
<td>95 ± 2%</td>
<td>94 ± 3%</td>
<td></td>
</tr>
</tbody>
</table>

Range observed 99–108% 90–100% 90–101%

Results

The patients' medical problems and medications did not appear to contribute to the results obtained for digoxin serum concentration. Serum protein concentrations were markedly abnormal in only one patient.²

Logit/log plots of displacement observed for the three standards and standard Laxolin are shown in Figure 1. Estimates in the range of 1.0–5.0 µg/liter are essentially similar for the four standards; those in the range of 0.25–1.0 µg/liter were more variable, especially in the Schwarz/Mann assay, largely because assay sensitivity is approached or exceeded. Results for patients' serum samples containing a high digoxin concentration diluted with Schwarz/Mann zero standard paralleled the Schwarz/Mann standard curve, whereas dilutions with digoxin-free serum did not (Figure 2).

Table 1 shows the percent binding, expressed as B/B₀, and the dose estimates; Table 2, the apparent recoveries based on those dose estimates. Nonspecific binding, estimated in the Schwarz/Mann assay for serum samples from the 24 patients receiving digoxin, was found to be 2.0 to 2.8% of the total activity, essentially identical to the usual assay nonspecific binding routinely measured for the zero standard (2.2% in the same assay run). Table 3 shows the binding estimates obtained in all three assays of the 39 sera from patients not receiving digoxin. Concentration estimates obtained by all three assays for the four control pools (Table 4) were respectively the same.

Discussion

Measurement of serum digoxin provides information that may result in a clinical decision to increase or decrease the amount of digoxin administered to a patient (6, 7). Underestimation of serum concentration may therefore be hazardous for the patient, because an increased dose may inadvertently produce toxicity. Some extrinsic factors, including serum albumin concentration (8, 9), serum thyroxine binding proteins (10), and the presence of drugs such as spironolactone (11), may affect the result obtained by radioimmunoassay. Moreover, characteristics of the assay performance itself may adversely influence the validity of the results. When an unknown patient dose estimate yields B/B₀ > 100%, there is concern regarding the validity of all dose estimates, and the assay performance should be evaluated. This phenomenon has been observed occasionally with the Schwarz/Mann charcoal-type assay by us and others (4). Davidson (4) suggested that this phenomenon might be explained by interference with the charcoal separation by a substance or substances unknown, with resulting misclassification of "free" as "bound" (4). Additionally, mis-standardization, nonspecific binding by proteins in the patient's serum, or differences between the incubational environment of the zero standard and patients' samples might explain this phenomenon.

Digoxin radioimmunoassay purports to measure total serum digoxin concentrations. Endogenous binding of digoxin to serum proteins and subsequent failure to measure this protein-bound fraction because of inefficient competition for binding by the assay antibody has been suggested as a possible cause of spuriously low results (12). The inclusion of 8-anilino-1-naphthalene sulfonic acid by one manufacturer (Abbott) to dissociate such endogenous binding led us to examine these specimens with that method.

In this study, digoxin concentration estimates (based on B/B₀ vs. dose) obtained with the Abbott and Cornning methods were higher in all samples than those obtained with the Schwarz/Mann charcoal assay (Table 2). In addition, only one instance (patient no. 10, Abbott) of B/B₀ > 100% was observed with the Abbott or Corning methods. These estimates correlate better with the amount of digoxin administered than the

² More detailed information regarding this patient is available from the authors.

496 CLINICAL CHEMISTRY, Vol. 24, No. 3, 1978
Table 4. Control Values (±1 SD)

<table>
<thead>
<tr>
<th>Assay kit</th>
<th>Control values, μg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O-I</td>
</tr>
<tr>
<td>Abbott</td>
<td>0.7 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>CV = 10%</td>
</tr>
<tr>
<td>Corning</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CV = 11%</td>
</tr>
<tr>
<td>Schwarz/Mann</td>
<td>0.8 ± 0.07</td>
</tr>
<tr>
<td>(B/B₀)</td>
<td>CV = 9%</td>
</tr>
<tr>
<td>Schwarz/Mann</td>
<td>0.8 ± 0.07</td>
</tr>
<tr>
<td>(B/T)</td>
<td>CV = 9%</td>
</tr>
</tbody>
</table>

CV = coefficient of variation. All are interassay, 5–7 runs.

O-I = Ortho Diagnostics I control sera.
O-II = Ortho Diagnostics II control sera.
CL = Control low digoxin pool.
CH = Control high digoxin pool.

apparently lower or even negative estimates obtained with the
Schwarz/Mann assay. All estimated concentrations were
<0.85 μg of digoxin per liter, approaching the sensitivities of
these assays.

Standardization in all three assays appears to be similar
(Figure 1). Underestimation of the standard concentration
would result in underestimation of an unknown dose. This
error should not result in B/B₀ >100%, however. Mis-standard-
ization does not appear to be a problem with any of the
three assays examined (Figure 1).

Although higher estimates were obtained with both poly-
edylene glycol and solid-phase separations than with the
charcoal separation, increased nonspecific binding by these
sera was not demonstrated and there was no evidence of in-
creased counts in the “bound” fraction due to misclassification
of “free” labeled ligand as “bound.” It does not appear that
there were substances present that interfered with the char-
coal separation. Dilution of high digoxin concentrations in
patients’ samples with Schwarz/Mann zero standard and with
a digoxin-free serum (Figure 2) also demonstrated these
binding differences. The “serum” used in the Schwarz/Mann
standard is actually defibrinated plasma, because large vol-
umes of human serum are not available to kit manufacturers.³

Apparent difference is sufficient to produce the ob-
served binding differences.

Recoveries from patients’ samples estimated by the Abbott
or Corning methods were generally acceptable. With the
Schwarz/Mann kit, apparent recoveries were high, being
higher for the high-value samples than the low-value samples;
this reflects inability to estimate digoxin present in the origi-
nal sample (Tables 1 and 2). Recoveries calculated when
the amount of digoxin present in the sample before addition was
based on B/T calculation were similar to those calculated for
the other two assays. The original apparently high recoveries
were due to the erroneous initial sample concentration esti-
mate, not to an assay-accuracy problem.

Measurements made with the Schwarz/Mann assay in sera
obtained from patients not receiving digoxin revealed that
binding in these samples was consistently greater than binding
in the zero standard (Table 3). This suggests that binding in
the zero standard is inappropriately low and appears to ex-
plain why B/B₀ >100% is also encountered in samples from
patients with low serum concentrations of digoxin.

Dose estimates made on four control pools were similar for
all three assays (Table 4). For dose estimate >0.8 μg/liter the
zero offset in the Schwarz/Mann standards does not appear
to lead to errors in concentration estimation.

Recalculation of the Schwarz/Mann data, omitting the zero
standard and estimating dose by relating “bound” activity/
“total” activity (B/T) to standard dose, resulted in dose es-
timates similar to the other two assays. This re-estimation of
dose in the patient sera also resulted in apparent recoveries
of 92–111%.

Protein concentration and drugs did not appear to be a
factor in this study. The Abbott method includes 8-anilino-
1-naphthalene sulfonic acid to inhibit endogenous digoxin
protein binding, and results with this assay in this group of
patients’ samples did not differ from those obtained with the
Corning assay or the recalculated (B/T) Schwarz/Mann
results.

The occurrence of samples with B/B₀ >100% observed with
the Schwarz/Mann charcoal assay appears to be related to
inappropriately low binding of labeled ligand in the zero
standard. Although this phenomenon appears to affect only
digoxin serum concentration estimates of <0.8 μg/liter, this
underestimation may result in inappropriate increases in
patient digoxin dosage. This effect can be corrected by esti-
mation of serum digoxin concentration by another method or
by calculating Schwarz/Mann results by using B/T and
omitting the zero standard.

We wish to recognize the helpful suggestions and review from Dr.
Albert Segaloff.

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³ Personal communication, Schwarz/Mann, Orangeburg, N.J.,
1977.
Adenylate Kinase Inhibition by Adenosine 5'-Monophosphate and Fluoride in the Determination of Creatine Kinase Activity

Franco Meliattini, Giuliano Giannini, and Paolo Tarl

The current methods for the determination of creatine kinase (EC 2.7.3.2) activity are derived from Oliver's method, in which AMP is used to decrease interference by adenylate kinase (EC 2.7.4.3). Recently, Szasz et al. and Rosano et al. described methods in which diadenosine pentaphosphate and fluoride, respectively, are used to reduce this interference. However, diadenosine pentaphosphate does not sufficiently inhibit such activity of hepatic origin, while fluoride alone can only inhibit it at concentrations at which the fluoride tends to precipitate as MgF₂. Finally, Szasz et al., the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology, and the German Society for Clinical Chemistry have proposed methods in which both AMP and diadenosine pentaphosphate are used to inhibit adenylate kinase. We have found that by using low concentrations of AMP and fluoride together, we can greatly diminish this interference without significant loss of creatine kinase activity and with no precipitation of MgF₂.

The usefulness of determining CK¹ activity as an aid to early diagnosis of myocardial infarction and progressive muscular dystrophy has already been well established (1, 2).

Present studies are chiefly aimed at resolving technical problems and at defining optimal assay conditions (3-6). However, the basic method for the determination of CK activity is still that described by Oliver (7), which includes the use of the reaction catalyzed by CK, in the reverse direction (the more favorable thermodynamically):

\[ \text{ATP} + \text{creatine} \rightleftharpoons \text{creatinine phosphate} + \text{ADP} \]

This is coupled with side reactions leading to the formation of substances that can be measured spectrophotometrically:

\[ \text{HK} \]

\[ \text{ATP} + \text{d-glucose} \rightleftharpoons \text{ADP} + \text{d-glucose-6-phosphate} \]

\[ \text{D-Glucose-6-phosphate} \]

\[ \text{G-6-PD} + \text{NADP}^+ \rightleftharpoons \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+ \]

However, the above-mentioned system is affected by AK activity (which is always present in normal sera, particularly in hemolyzed samples), which catalyzes the formation of ATP according to the reaction:

\[ 2\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP} \]

Therefore, the presence of AK causes an apparent artificially high value for CK activity.

Oliver (7) and others (3, 8) noticed that AK could be inhibited by AMP in concentrations five- to 10-fold that of ADP. However, at these concentrations, AMP also slightly inhibits CK activity (9), and this effect cannot be ignored. Later, some modifications were proposed, in which AMP was used in lower concentrations in order not to inhibit CK appreciably while causing sufficient, even if incomplete, inhibition of AK. Thus, the concentration of AMP was decreased from 20 mmol/liter to 10 mmol/liter (3) and then to 5 mmol/liter (6). However, with an AMP concentration of 5 mmol/liter CK is inhibited little, but the AK is not sufficiently inhibited (6, 9).

Recently, Rosano et al. (9) proposed the use of fluoride, while Szasz et al. (10), the Scandinavian Committee on Enzymes (11), and the German Society for Clinical Chemistry (12) suggest the use of both AMP and diadenosine pentaphosphate.

We studied the possibility of using AMP and fluoride together, and found that is possible to inhibit AK effectively with very low concentrations of them. Under the proposed conditions, the inhibition of CK is negligible and the precipitation of fluoride is avoided.

Materials and Methods

Creatine phosphate, ADP, and AMP were from Prochifia, Milan, Italy; HK (from yeast) and G-6-PD (from Leuconostoc mesenteroides) were from P&L Biochemicals Inc., Milwaukee.